Mid-size Insert: 8-10 kb Library Creation Protocol

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Author: Chris Detter, Eileen Dalin
Reviewed by: Jamie Jett, Doug Smith
Approved by: Paul Richardson, Chris Detter

Summary

To construct a randomly sheared, non-biased library containing 8-10 kb inserts.

Subcloning---Materials & Reagents

Materials/Reagents/Equipment	Vendor	Catalog Number
Disposables		
Phase Lock Gel	Eppendorf	0032 005.101
Clear Round Bottom 2 ml tubes	Eppendorf	22 36 335-2
Reagents		
10X Eco Pol Buffer	New England Bio Labs	B9006S
10mM Nucleix Plus dNTP	Amersham	US-77212
T4 DNA Polymerase (100U 1U/uL)	Roche	1004786
Klenow Large Fragment	New England Bio Labs	M0210L
Low Melting Point Agarose	Invitrogen	15517-022
AgarACE	Promega	M1743
Phenol	Sigma	P4557
5M NaCl	Ambion	9759
Pellet Paint	Novagen	69049-3
100% EtOH		
P21 vector	JGI	
Polyethylene Glycol	Sigma	P-5413
T4 DNA Ligase	Roche	79909
10X ligation buffer	Roche	included
Equipment		
Hydroshear	Gene Machines	
Hydroshear Large assembly	Gene Machines	HSH 204007
Syringe	Gene Machines	HAS-S
Hydroshear Wash Kit	Gene Machines	HSH-KT1
-80C freezer		
-20C freezer		
Juan vacuum centrifuge		
Microcentrifuge (13,500 rpm)		

Procedure

Shearing:

1. Transfer to **well-labeled** Eppendorf tube 5 to 10 µg of non degraded DNA; Final volume 100 µl.
   Dry 200 µl *of DNA down to 100* µl *needed*

2. Shear with large assembly.
   a. Volume = 100 µl
   b. # of cycles = 25 cycles
   c. Speed code = 9 (may vary by source of DNA)

3. Collect sample and place on ice until all samples are complete

Blunt End Repair:

1. To 95 µl of sheared DNA, add:

	1 Rxn
10x Klenow Buffer ----------	13 µl
10mM dNTPs ----------	10 µl
T4 DNA Polymerase (1U/µl) ---------	6 µl
Klenow Fragment (5000U/mL) ------	6 µl
	35 µl

   ** Make master mix if needed, keep on ice.

2. Cap **well-labeled** tube, Vortex, and Spin Down.

3. Incubate:
   a. RT for 40 minutes
   b. 70°C for 15 minutes
   c. 4°C for 10 minutes
   d. Freeze at –20°C for 30 min before running gel or store overnight @ –20°C

Size Fractionation/Gel Separation:

Pulse Field Conditions:

a. % Agarose---- 1.0% Low Melting Point Agarose
b. Buffer---------- 0.5x TBE
c. Temperature-- 14^0C
d. Voltage--------- 6 V/cm
e. Pulse---------- 2.0-4.0 sec
f. Run Time----- 15 hrs
g. Angle---------- 120^0

1. Add 10-20 µl loading dye to sample.
2. Load onto 1% LMP agarose gel (0.5x TBE).
3. Run gel overnight with above parameters (run with size “Marker 2”).
4. Remove gel from pulse field platform.
5. Stain gel with an Ethidium bromide solution in distilled water (0.5 µg/ml) for 30 minutes.
6. Destain gel in distilled water for 1.5-2 hours.
7. Cut out 8-10kb band and place in **well-labeled** 2 ml round bottom eppendorf tube.

Gel Digestion:

1. Place tubes at 65°C to melt gel **completely** (approximately 5 to 20 minutes).
2. Place at 42°C to equilibrate for 3 to 5 minutes.
3. Add 6-8 µl AgarACE to each tube.
4. Mix well and Spin down.
5. Incubate at 42°C for 20 minutes.

Phenol Extraction:

1. Prepare phase lock tubes, spin at 10,000 RPM for 2 min.
2. Measure sample volume.
3. Add an equal amount of phenol (~1 ml).
4. Vortex well for 15-30 sec.
5. Add to **well-labeled** phase lock tubes.
6. Spin tubes for 5 minutes at 10,000 RPM.
7. Pull off (top) aqueous layer into **well-labeled** 2 ml round bottom tube.

EtOH ppt:

1. Measure sample volume.
2. Add 1/10 volume of 1M NaCl, 1.5 µl pellet paint, and 2.5 volumes of 96% EtOH.
3. Vortex well and spin to collect.
4. Place at –80°C for at least 30 minutes.
5. **Pre-chill microcentrifuge to 4ºC,** this takes at least 15 min.
6. Spin at 13,500 rpm for 20 minutes at 4°C.
7. Dump off supernatant-Discard, keep an eye on the pink pellet.
8. Wash pellet with 200 µl 96% EtOH.
9. Pull off supernatant being careful of “the wiley pellet”.
10. Dry pellet in vacuum for 5 min. at medium heat (combine pellets after drying if needed).
11. Resuspend pellet in 22 µl T0.1E; vortex and spin down sample.
12. Place at 50°C for 5 minutes to fully resuspend pellet.
13. QC 2 µl of sample on 1% agarose gel for size and concentration.

Ligation:

1. Make up p21/10X/T4 ligation buffer cocktail.

1x
0.3 µl p21
0.8 µl 10X Buffer
0.6 µl T4 DNA ligase
0.7 µl H_2O
2.4 µl

** Make master mix if needed, keep on ice.

2. Add **4.4 µl** of purified DNA to the bottom of a clean, **well-labeled** 1.5 ml tube.
3. Aliquot **2.4 µl** of ligation cocktail to the bottom of the tube.
4. Add **1.2 µl** of 30% PEG to the bottom of the tube.
5. MIX WELL, spin to collect.
6. Incubate overnight at 16°C.

Phenol Extraction:

1. Prepare phase lock tubes, spin at 10,000 RPM for 2 min.
2. Bring sample volume up to 50 µl with T0.1E (42 µl).
3. Add an equal volume (50 µl) of phenol.
4. Vortex well for 15-30 sec.
5. Add to **well-labeled** phase lock tubes.
6. Spin tubes for 5 minutes at 10,000 RPM.
7. Pull off (top) aqueous layer into clean, **well-labeled** 1.5 ml tube.

EtOH ppt:

1. Add 1/10 volume 1M NaCl, 1.5ul pellet paint, and 2.5 volumes of 96% EtOH.
2. Mix well, spin to collect.
3. Place at –80°C for at least 30 minutes.
4. **Pre-chill microcentrifuge to 4ºC,** this takes at least 15 min.
5. Spin at 13,500 rpm for 20 minutes at 4°C.
6. Dump off supernatant-Discard, keep an eye on the pink pellet.
7. Wash pellet with 200 µl of 96% EtOH.
8. Pull off supernatant being careful of “the wiley pellet”.
10. Dry pellet in vacuum for 5 min. at medium heat.
11. Resuspend pellet in 20 µl T0.1E; vortex and spin down sample
12. Place at 50°C for 5 minutes to fully resuspend pellet.
13. Transform immediately or store at -20ºC.

Transformation---Materials & Reagents

Materials/Reagents/Equipment	Vendor	Catalog Number
Disposables		
Gene Pulse Cuvette 0.1 cm electrode gap	BioRad	165-2089
Falcon 14mL Polypropylene tube	Becton Dickinson	352059
Cryogenic Vial	Corning	430289
LB KAN 30 X-Gal Plates	Teknova	L4908
Reagents		
ElectroMAX DH10B Cells	Invitrogen	18290015
SOC Medium	Teknova	0166-10
Equipment		
-80C freezer		
-20C freezer		
Gene Pulser II	BioRad	
Pulse Controller Plus	BioRad	

Transformation:

Equipment Settings (BioRad Pulse Controller):

- Low range: 200
- High range: ∞
- Capacitance: 25
- Voltage: 1.8 kV

1. Place on ice: **well-labeled** eppendorf tube and cuvette.
2. Thaw ElectroMAX DH10B competent cells on ice.
3. To the appropriately labeled, COLD Eppendorf tube; add 2 µl of ligation product.
4. Once thawed, mix competent cells by **swirling** with pipette tip a few times
5. Add 50 µl eDH10B competent cells to the Eppendorf tube containing ligation.
6. Mix by **swirling** with pipette tip a few times
7. Transfer solution to the bottom groove of the COLD cuvette and **tap** on tabletop a few times to settle solution to the bottom (no bubbles).
8. Electroporate at 1.8 kV.
9. Transfer cell solution IMMEDIATELY to 950 µl of RT SOC **(make sure SOC is clear, i.e. no growth)**.
   (Transfer electroporation within 10 seconds.)
10. Rinse cuvette with 50 µl of the same SOC mixture you just added the cells to.
11. Incubate within rotating wheel at 37 ^{o}C for 1 hour.
12. After 1 hour incubation, place transformation on ice (no longer than one hour) until ready to plate on agar plates.

Plating:

1. Before the 1 hour is up, prepare one **well-labeled** LB/KAN 30 x-gal (30 µg/ml) agar plate per library by letting them warm to 37°C in an incubator to dry.
2. After 1 hour, plate ~30 µl of transformation glycerol mixture onto the appropriately labeled bioassay.
   - First pipette ~ 500 µl SOC onto the center of the plate, then add 30 µl of transformation to the center with the SOC.
   - Spread in a small circular motion at first to help mix, and then spread evenly across the entire plate.
3. Then make a 10% glycerol transformation stock (139 µl 80% glycerol + remaining transformation (970 µl = ~ 1109 µl gly. trans. stock). Cap, then mix by inverting several times.
4. Store transformation glycerol mixture immediately @ -80 °C.
5. Incubate the plates in 37°C incubator for 16-18 hrs.
6. Count colonies and determine the complexity of ligation reaction (total # of colonies in ligation).

Insert size QC---Materials & Reagents

Materials/Reagents/Equipment	Vendor	Catalog Number
Disposables		
96 well PCR plate		
Reagents		
Templiphi 10000 rxn kit	Amersham	25-6400-01
Denaturation Buffer	Amersham	included
SWA I	New England Biolabs	R0604L
10X Buffer 3	New England Biolabs	included
100X BSA	New England Biolabs	included
Equipment		
Thermocycler PE9700		

Rolling Circle Amplification:

Denaturation

1. Dispense 10 µl of 1x Denaturation Buffer into 96 well sample PCR plate.
2. Place in centrifuge for quick spin down.
3. Make a visual CHECK to make sure all plates have buffer.
4. Using disposable tips, pick 1 colony (white) into each well.
5. Mix and carefully pull out tips.
6. Place in centrifuge for quick spin down.
7. Seal plate with Robbins plate seal.
8. Place plate on thermocycler for 5 min at 95ºC.
9. After 5 min denaturation, immediately place plate on ice.
10. Cool on ice for at least 5 minutes.

Amplification

1. Remove RCA mix from -80°C freezer before initial denaturation to allow it to thaw on ice.
2. Gently mix when thawed.
3. Dispense 10 µl of RCA mix into each well of the sample PCR plate.
4. Place in centrifuge for quick spin down
5. Make a visual check that all plates have RCA mix
6. Seal plate with Robbins plate seal
7. Place 96 well PCR plate into thermocycler
8. PCR program: 30ºC for 20 hours; 65ºC for 10 min; 4º hold

Digestion

1. Aliquot 5 µl of RCA product into a 96 well PCR plate.
2. Spin down plate.
3. Make a visual check that all wells have RCA product.

4. Make digestion cocktail:

	1x	120x
10X buffer	1.0 µl	120 µl
100X BSA	0.1 µl	12 µl
SWA I	1.0 µl	120 µl
H_2O	2.9 µl	348 µl
	5.0 µl	600 µl

5. Dispense 5 µl of digestion cocktail into each well
6. Seal top, Vortex & spin down plate
7. PCR program: 25ºC for 4 hours; 65ºC for 20 minutes; 4ºC hold
8. Add 10 µl loading dye to samples
9. Vortex and spin down samples
10. Load 15 µl of digested RCA product onto a 1% agarose gel with size standards (Save the remaining 5 µl to use in case there is a problem with the QC gel. Can toss after gel is imaged).
11. Run for ~30 minutes at 120V.
12. Check for inserts at 8-10 kb

Reagent/Stock Preparation

T0.1E

40 µl 0.5M EDTA
2 ml 1M Tris-HCl
197.960 ml H_20
pH 8.0

30% PEG

3 g Polyethylene Glycol
10 ml H_20